Saskatchewan
Agriculture, Food
and Rural
Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

19980111

THE APPLICATION OF BIOLUMINESCENT AND GREEN FLUORESCENT PROTEINS TECHNOLOGY TO TRACK AND EVALUATE VIABILITY OF PROBIOTIC FLORA IN PIG AND POULTRY

Funded by: The Agriculture Development Fund

October, 2002
Prepared by: University of Saskatchewan

105-00366/

Final Report
Saskatchewan Department of Agriculture and Food
Research Project

Project: 98000111
Entitled: "The application of bioluminescent and green fluorescent proteins technology to track and evaluate viability of probiotic flora in pigs and poultry"

Abstract:

Evaluation of the efficacy of potentially probiotic bacterial strains is often limited by the ability to determine if the strain under study effectively colonizes the intestine and the relationship between probiotic colonization and colonization by potential harmful bacteria. This research activity was designed to evaluate green fluorescence and bioluminescence recombinant "signaling" technology to track intestinal bacterial colonization in pigs and chickens. We reported previously the attempt to insert the GFP gene using the same transposon into *Lactobacillus delbreukii* and *Lactobacillus acidophilus*. The insertion was observed by the fluorescence of the lactobacilli cells, however, by subculturing the bacteria, the GFP gene appears to be repressed or lost and no further fluorescence was detected, thus, no animal studies could be carried out to track the colonization of the lactobacilli. For the continuation of our studies, tracking the intestinal colonization of probiotic strains, we have elicited specific antibodies to lactobacilli and bifidobacteria to use as probes in intestinal sections. It is hoped the use of these antibodies will provide an efficient system for tracking the colonization of bacteria following inoculation of poultry and swine.

Executive Summary:

Due to the negative publicity of antibiotics in animal production, other alternative practices are being sought to improve animal health and performance. One approach may be to feed beneficial bacteria (i.e., probiotic organisms) that alleviate gastrointestinal problems and promote well-being in animals. In the last decade, research of the microbial composition in animal intestines has been recognized as a major contribution to animal welfare and growth performance. Furthermore, some probiotics have made their way to the commercial marketplace. However, much of the available research in probiotics has yielded variable and/or inconclusive results. For example, success of treatment is often judged by obscure indirect evaluations using selective culturing media (i.e., microorganisms) and/or feed efficiency and growth performance in animal feeding trials. Since indirect methods cannot determine the degree, location and duration of bacterial colonization in the intestine, scientists rarely determine if the probiotic strain administered successfully colonized the animal's intestine, the location of that colonization and/or if harmful (pathogenic) bacteria have been excluded.

The present investigation was proposed to examine the application of "signaling" technology to assess probiotic colonization in the gastrointestinal tract of birds and pigs. The successful development of this technology will allow scientists to determine directly the degree, location and duration of colonization of probiotic flora under various nutritional and management conditions.

Initially, priority was given to show the utility of the green fluorescence approach. We previously reported the attempt to insert the GFP gene using the same transposon into *Lactobacillus delbreukii* and *Lactobacillus acidophilus*. The insertion was observed by the fluorescence of the lactobacilli cells, however, by subculturing the bacteria, the GFP gene appears to be repressed or lost and no further fluorescence was detected, thus, no animal studies could be carried out to track the colonization of the lactobacilli.

A primary objective of this project is to visualize probiotic and pathogenic bacteria within the intestinal mucosa. In order to successfully track the colonization of bacterial strains in the intestinal mucosa, we previously reported a preliminary study where the colonization of *Salmonella enteritidis* was effectively visualized by the use of fluorescent-labelled antibody probes. Using confocal laser microscopy we visualized salmonella in the cecal mucosa of chickens. We have raised in rabbits specific antibodies to lactobacilli and bifidobacteria, which are being used to track the colonization of probiotic and pathogenic bacteria following inoculation of poultry and swine. We have performed initial trials using germ-free chickens that were infected with *S. enteritidis*, bifidobacteria and lactobacilli strains. A recent gnotobiotic experiment was performed to evaluate the colonization patterns of *S. enteritidis* and the probiotic *Lactobacillus fermentum*, unfortunately, due to poor hatchability of the chickens inside the isolators, some groups of chickens which were intended to be studied could not be accounted for. Of some groups of chickens, tissues samples were obtained and are being analysed on their colonization patterns by scanning confocal microscopy using the specific antibody fluorescent-labelled probes. We expect that in an oncoming trial, using this tool, the interaction of pathogens such as *S. enteritidis*, *Campylobacter jejuni* and probiotic bacterial strains of lactobacilli and bifidobacteria can be further investigated in pursuit of the identification of the colonization factors involved, thus contributing to our understanding of the interaction among bacteria in the intestinal tract.

Technical report:

For the Period March 1, 2001 to August 31, 2001

Objectives

1. To determine the use of specific antibody probes against *Salmonella enteritidis*, *Campylobacter jejuni*, lactobacilli and bifidobacteria to study their colonization within the microenvironment of intestinal mucosa.
2. To test the suitability of Scanning Confocal Laser Microscopy (SCLM) to study intestinal bacterial colonization.
3. To elucidate the spatial colonization patterns of pathogen-probiotic bacteria within the gut ecosystem.

Materials and Methods

Development of specific antibody fluorescent probes for tracking of intestinal colonization

Lactobacilli strains (*L. fermentum, L. acidophilus, L. salivarius, L. delbreukii, L. amylovorus* and *L. reuteri*) and bifidobacteria strains (*B. longum, B. thermophilus, B. gallinarium, B. pullorum, B. choerinum, B. adolescentis, B. animalis, B. pseudolongum, B. suis* and *B.bifidum*) were grown in trypticase soy broth (TSB) for 24 hours at 37 ^{0}C. *Salmonella enteritidis* was grown in TSB for 24 hours at 37 ^{0}C and *Campylobacter jejuni* was grown in the same medium for 48 hours at 37 ^{0}C. The lactobacilli and bifidobacteria strains were pooled. All the different bacteria were centrifuged at 1000 x *g* and the pellets were washed by centrifugation twice and resuspended in PBS to a final concentration of 10^9 bacteria per ml. The bacteria were converted into bacterins by the addition of 0.6 % formaldehyde. Five-hundred µl of each bacterin was adjuvanted with an equal amount of mineral oil and the emulsion was injected subcutaneously in rabbits. The animals were boosted with the bacterins 5 times. The serum antibodies were collected, concentrated and affinity chromatography purified. Serum antibody levels specific for lactobacilli, bifidobacteria, *S. enteritidis* and *C. jejuni* were evaluated by an ELISA test.

Salmonella enteritidis and Lactobacillus fermentum infection

One trial where 20 sterile eggs were set inside each of three germ-free isolators was carried out. The hatchability in the isolators was poor, and no chicken was hatched in isolator 2 which would be infected with *Salmonella enteritidis* alone. In isolator 1, infected with *Lactobacillus fermentum* alone hatched 7 healthy chickens, while in isolator 3, infected with *Salmonella enteritidis* and *Lactobacillus fermentum* produced 5 healthy chickens. A germ-free environment was produced following standard protocols for incubating, feeding, watering and maintaining birds under germ-free conditions for 20 days from hatching. Fertilized chicken eggs were incubated for 18 days, and then they were candled and disinfected. Viable eggs were introduced into a germ-free isolator where they were hatched. Autoclaved food and water were available *ad libitum*. *S. enteritidis* and *L. fermentum* were cultured for 18 hours in TSB. The chickens were infected by oral gavage with 10^5 *S. enteritidis* and 10^8 *L. fermentum* in 500 µl of TSB, respectively. The chickens were euthanized on day 20 post-infection. One cm intestinal sections were obtained from the jejunum, ileum and cecum preserved in Cryomatrix embedding resin, snap frozen in liquid nitrogen and stored at –80^{0}C until analysis.

Experimental Protocol:

		Experimental Days									
Isolator	*Group*	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8-19*	*20*
1	Lactobacillus	L_1 H	L	L	L	L	L	L	L	-	E
2	Salmonella	S H	-	-	-	-	-	-	-	-	E
3	Salmonella/Lactobacillus	L_1 H	L S	L	L	L	L	L	L	-	E

10 chickens/group.

H – hatching.

L_1 – *Lactobacillus fermentum* application to feed – ~10^9 Lactobacillus.

S - *Salmonella enteritidis* application to feed – ~10^6/chicken.
L – *Lactobacillus fermentum* in water – 10 mLs of a ~10^7/mL culture.
E - euthanasia and sampling for SCLM (ileum and cecum) and *Salmonella/Lactobacillus* counts (ileum, cecum, liver and spleen).

Intestinal and tissue S. enteritidis and L. fermentum counts

Intestinal contents, spleen and liver tissue were placed in pre-weighed 15 ml sterile culture tubes containing 1 mL peptone water. Spleen and liver tissues were macerated using a polytron apparatus. The intestinal contents and spleen and liver homogenates were thereafter diluted in peptone water to appropriate plating dilutions. For *S. enteritidis* counts, diluted samples were plated on Brilliant Green Sulfa agar containing 25 µg/ml naladixic acid and novobiocin using an automated plater. For *L. fermentum* counts, diluted samples were plated on MRS agar. Plates were cultured 18 hours at 37^{0}C under aerobic conditions and counted to determine colony forming units (cfu) per gram of intestinal contents or tissue.

Tissue preparation and analysis by scanning confocal laser microscopy

Frozen intestinal samples were placed at -20°C for 1 hour prior to sectioning. 30 µm tissue sections were obtained using a Cryo-cut II Cryostat Microtome (American Optical, Buffalo, NY, USA). Sections were placed in a 12 well tissue culture plate for all washing and antibody labeling. Labeling with an anti-salmonella or anti-lactobacillus primary antibody was done overnight at 4°C. Samples were then washed in PBS for 1.5 hr with two changes of PBS. Secondary labeling was done with a goat anti-rabbit FITC conjugated antibody for 1.5 hrs, and rinsed in PBS for 1.5 hrs with two changes of PBS. All rinses and secondary labeling were done at ambient room temperature. Labeled tissue was mounted on a coverslip prior to observation. Intestinal tissue analyses were conducted with a Bio-rad MRC 600 lasersharp fluorescence scanning microscope mounted on a Nikon FXA microscope equipped with a 60 X plan-apochromatic objective lens. The laser light source used for the excitation of the fluorescently labeled antibodies is an argon lamp (maximum emission lines at 488 and 514 nm). A Northgate 480 desktop computer equipped with Bio-rad software was used for the collection and processing of the digital images. Optical thin sections of intestinal tissue samples were collected at 1 µm increments through the sample's z axis using the microscope motorized focus control operated through the Bio-rad software. Presented images were composite projections of a number of the collected optical thin sections.

Results

Figure 1 shows the number of *S. enteritidis* and *L. fermentum* in the ileum, cecum, spleen and liver, 20 days after oral challenge. Colonization for both bacteria occurred in the intestine with greatest numbers observed in the cecum. Figure 2 shows the number of *L. fermentum* in the ileum, cecum, spleen and liver, 20 days after oral challenge. Colonization occurred in the intestine with greatest numbers observed in the cecum.

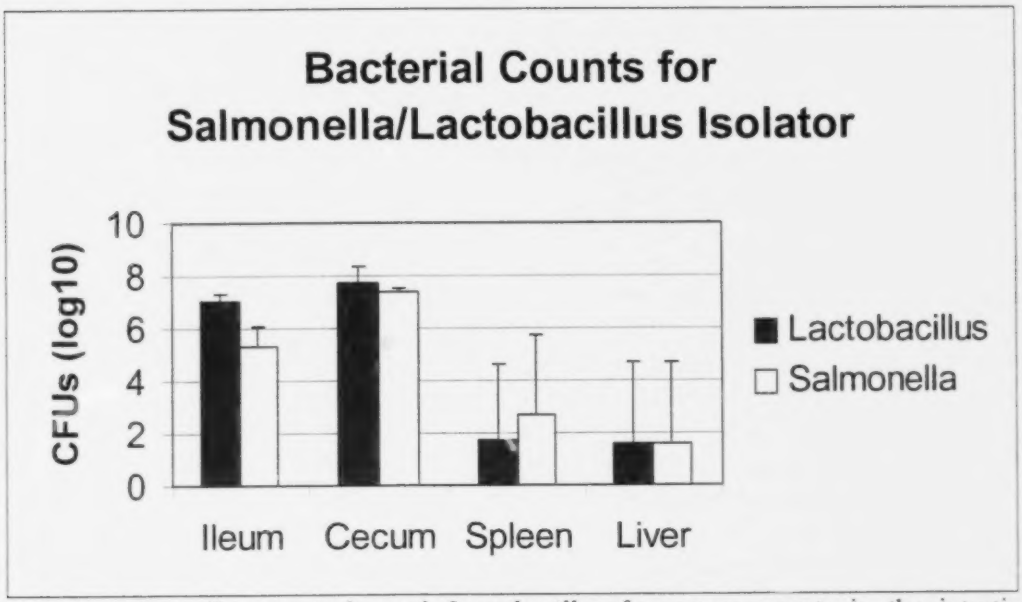

Fig. 1. *Salmonella enteritidis* and *Lactobacillus fermentum* counts in the intestinal contents (ileum and cecum) and in spleen and liver of gnotobiotic chickens 20 days after oral infection. Bars represent the means of colony forming units (CFU) per gram of intestinal material or tissue ± SD.

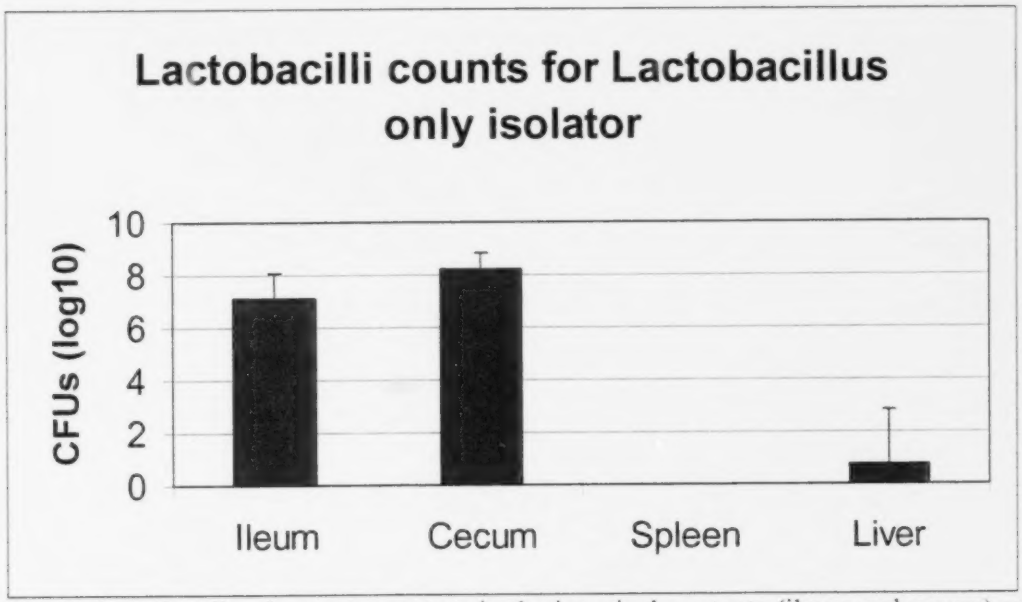

Fig. 2. *Lactobacillus fermentum* counts in the intestinal contents (ileum and cecum) and in spleen and liver of gnotobiotic chickens 20 days after oral infection. Bars represent the means of colony forming units (CFU) per gram of intestinal material or tissue ± SD.

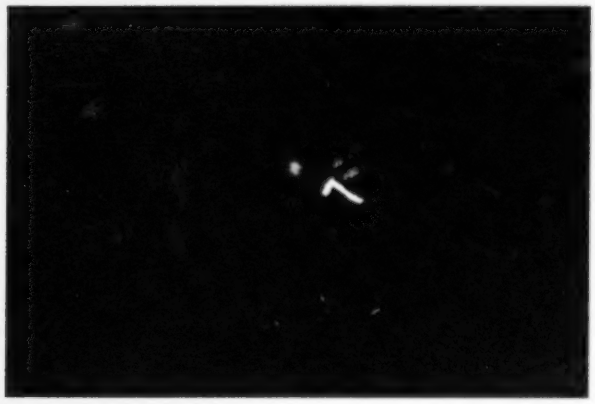

Fig. 3. Optical sectioning of ileal tissue, on experimental day 20, produced by a confocal laser scanning microscope, illustrating the colonization of *Lactobacillus fermentum* at different flow cell depths.

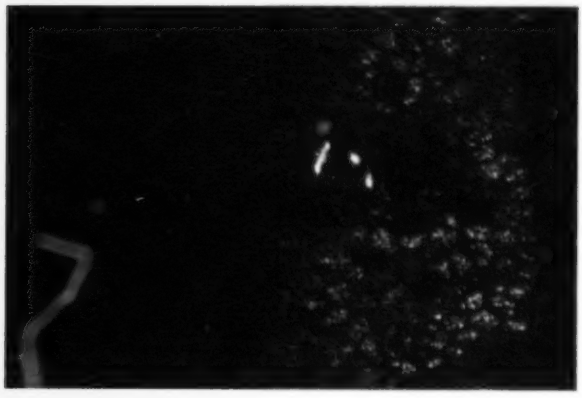

Fig. 4. Optical sectioning of cecal tissue, on experimental day 20, produced by a confocal laser scanning microscope, illustrating the colonization of *Lactobacillus fermentum* at different flow cell depths.

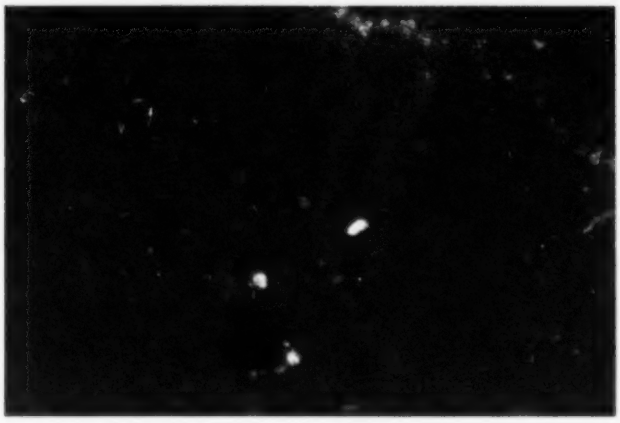

Fig. 5. Optical sectioning of ileal tissue, on experimental day 20, produced by a confocal laser scanning microscope, illustrating the colonization of *Salmonella enteritidis* at different flow cell depths.

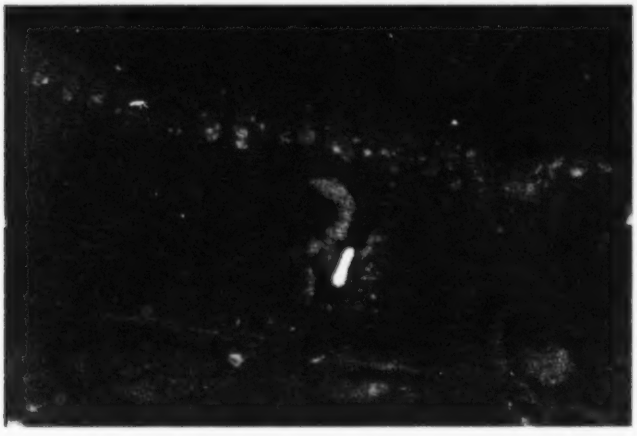

Fig. 5. Optical sectioning of cecal tissue, on experimental day 20, produced by a confocal laser scanning microscope, illustrating the colonization of *Salmonella enteritidis* at different flow cell depths.

Discussion

This study using anti-*S. enteritidis* and anti-lactobacilli fluorescein-labeled antibodies in conjunction with scanning confocal laser microscopy (SCLM), indicates that these techniques may provide a novel and informative approach for the study of bacterial colonization leading to the development of successful intervention strategies against pathogen and probiotic bacteria. The use of SCLM as a tool for the study of intestinal colonization is limited for the time being, however, we have observed detectable and distinct colonization patterns of bacteria both in conventional and in gnotobiotic chickens. In this reported study, the overall goals were marred by the poor hatchability of the experimental chickens, which did not allow having the experimental groups that were going to be studied on the colonization of pathogen and probiotic bacterial strains. Of the groups that could be studied – *S. enteritidis-L. fermentum* and *L. fermentum* alone, it was possible to identify their colonization patterns on the intestinal epithelium. *S. enteritidis* was found to penetrate deeper into the cecal epithelium than *L. fermentum* which appeared to be more loosely associated with the epithelium, possibly in closer association with the mucus layer, however, as the technique employed does not permit the preservation of the mucus layer, the association can not be confirmed. Better methods for the fixation, staining, and mounting of tissue may allow the overlying mucous layer to be preserved, thus permitting a more comprehensive understanding of the ecological or spatial niche that pathogens occupy. The evaluation of the ecological niches occupied in the cecal epithelium by microorganisms is of importance for the selection of probiotic strains, for example, a tightly associating probiotic strain to the epithelium will likely be effective against samonella, while a probiotic strain whose niche is the mucus layer will be more effective for the control of campylobacter. We are in the process of evaluating better fixation methods, which will preserve the mucus layer and will allow us to visualize the spatial relationships of mucus-dwelling bacteria such as campylobacter and other probiotic strains. We will be performing a gnotobiotic trial in which we will infect germ-free chickens with campylobacter and a lactobacillus or bifidobacterium strain and will evaluate their spatial colonization on the epithelium and in the mucus layer. To accomplish these studies, we request an extension of the project to provide SADF a final report.

Personnel

The technical salary of Mr. Jason Marshall the 4 month period from March 1, 2001 to June 30 20001 was provided by project funds.

Equipment

N/A.

Project Developed Materials

N/A.

Project Photos

N/A.

Acknowledgement

At this time there have been no presentations or publications related to this project. The Saskatchewan Agriculture Development Fund will be acknowledged in such cases.